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We have purified a low affinity form of estrogen receptor (type II estrogen binding site, EBS) which is distinct in its steroid and DNA binding properties from the type I estrogen receptor. Purified type II EBS was utilized to generate polyclonal antibody against this protein in mice. The antibody was able to detect the type II EBS in Western Blot. In order to identify the gene encoding the type II EBS, a cDNA library was created in λgt11 using cDNA prepared from pregnant rat uterine tissue. The library was subsequently screened using the type II EBS antibody prepared in mouse. Although two putative clones were initially isolated, they were determined to be false positives following subsequent purification and re-screening. These results seem to indicate that the antibody was of insufficient titer for use in screening the uterine cDNA library. It is possible that rat type II EBS displays low level immunogenicity in mice. Future goals for this project include: induction of expression of type II EBS in mice using estrogen implants, followed by the development of a higher yield purification procedure, which should aid in the identification of the type II EBS gene.

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FOREWORD

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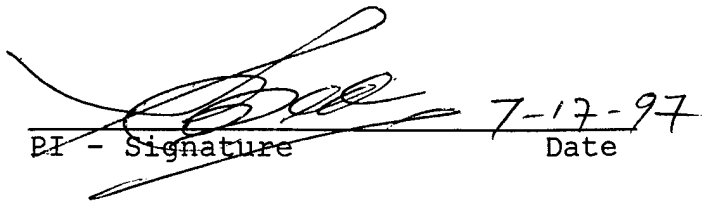

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INTRODUCTION

The type II ER level has been shown to be high in estrogen-dependent mouse mammary tumors, human breast cancer, and certain cell lines, suggesting a possible role in these tumors. However, its apparent presence in normal tissue, such as rat uterus is observed only with exogenous administration of estradiol. The administration of estradiol to rat results in the stimulation of uterine growth as well as the level of type II ER. Thus, type II ER may be involved in growth of the uterus and perhaps uterine cell proliferation. Furthermore, its presence in various endometrial cancer and different cancer cell lines has led to the hypothesis that the type II ER may play a crucial regulatory role in estrogen stimulated tumors. In order to determine the precise role that type II ER plays in these processes, the receptor should be purified and its gene cloned.

Despite the rapid advancements in the biochemical and genetic characterization of type I ER, very little progress has been made in elucidating the precise structure and function of type II ER. The limited physicochemical information which is available on type II ER has been obtained on crude preparations. Type I ER is used as an indicator of the estrogen responsiveness of a tissue. In addition, the type I ER is used as a prognosis factor for breast cancer. However, it is becoming clear that the effects of estradiol cannot be explained only through its interaction with the type I ER. To begin to assess the role the type II ER may play in estrogen action, we have purified the receptor to near homogeneity and its biochemical properties have been characterized. Purification of the type II ER and identification of its gene will aid in the determination of the precise role that type II ER plays in these processes.

BODY OF REPORT

SYNOPSIS OF YEAR 01 RESULTS

A purification for near homogeneous preparations of type II ER (henceforth referred to as estrogen binding site (EBS) was developed and its biochemical properties were characterized. The details of these studies are described in, Grey, W.G.N., et al. (1994) A low-affinity estrogen-binding site in pregnant rat uteri: Analysis and partial purification. *Proc. Natl. Acad. Sci, USA*, 91, 11502-11506 (1). The abstract from the article is given below.

We have identified a low affinity (type II) estrogen binding site (EBS) which is expressed at high levels during pregnancy in rat uteri. Although this activity was detectable in non-pregnant rat uteri, it was present in amounts (0.094 pmol/g of uteri) which were several fold lower than the high affinity type I ER (0.57 pmol/g of uteri). During pregnancy, at 19-20 days gestation, the low affinity type II EBS became the major ($\geq$88%) estrogen binding site in rat uteri. The increase in the level of low affinity EBS (7.9 pmol/g) in uteri was $\sim$85-fold with a $\sim$20-fold increase in the specific

activity (0.39 pmol/mg) of this form, while the high affinity form remained relatively unchanged.

We report here a method of purification of type II EBS from pregnant rat uteri and presented an analysis of its DNA and steroid binding properties. Estradiol binding studies and Scatchard analysis demonstrated that the type II EBS had an apparent estradiol binding affinity of ≥24 nM. Gel filtration and SDS-PAGE analysis indicated that the type II EBS was a monomeric protein with a molecular weight of 73 kDa. The estradiol binding remained apparently uninhibited in the presence of a large excess of tamoxifen, nafoxidine, or dihydrotestosterone. Estradiol, DES and quercitin (a type II EBS specific inhibitor) competed efficiently. The purified low affinity EBS did not have sequence specific DNA binding activity with the estrogen responsive element (ERE) which indicated that it is different in function from the type I ER.

SYNOPSIS OF YEAR 02 RESULTS

PREPARATION OF TYPE II EBS POLYCLONAL ANTIBODY

Due to the limited quantities of purified protein, mice were selected to generate antibody against the type II EBS. The procedure was as follows; antigen-adjuvant emulsion was prepared using purified type II EBS (Fr V) and Ribi (Hamilton, MT) (2) adjuvant. This adjuvant was selected due to its low level toxicity and ability to potentiate a good immune response with low levels of antigen. Six week old, female balb-C mice were each immunized with emulsion containing 5 μg of EBS, subsequent boosting was carried out according to the manufactures suggestions. Sera were tested for cross-reactivity to type II EBS by their ability to recognize the polypeptide in Western blot and by their ability to inhibit estrogen binding *in vitro*. Although the antibody was able to recognize the EBS peptide in Western blot (Figure 1), it was unable to inhibit estrogen binding *in vitro*. This could be due to a variety of reasons, including low titer or simple failure to generate antibody against epitopes critical to estrogen binding. However, this antibody was deemed to be suitable for the screening of a rat uterine cDNA/genomic DNA library as it appeared to recognize the EBS protein.

PREPARATION OF THE RAT UTERINE λgt11 cDNA LIBRARY

As no pre-made rat uterine cDNA libraries were commercially available, it was necessary to construct one in the laboratory. cDNA was prepared using the Great Lengths cDNA System (Clonetech; Palo Alto, CA) and pregnant rat uterine tissue. The rat cDNA library in λgt11 was prepared following the methods described by Huynh, et al (3).

SCREENING THE RAT UTERINE cDNA LIBRARY

Screening the rat uterine cDNA library in λgt11, described above, was carried out using type II EBS antibody, following the procedure of Huynh, T. et al (3). *E. coli* strain Y1090 (0.2 ml) was infected with 0.1 ml of lambda phage containing 3 X 10^4 pfu in top agar and plated onto 100 mm plates. The plates were incubated at 42°C for 3.5 hours following which they were overlaid with IPTG impregnated nitrocellulose filters and transferred to 4°C. Antibody against type II EBS was then used to probe the replica filters, using horse radish peroxidase conjugated second antibody and chemi-luminescent detection. Initially two putative clones were obtained, but were determined to be false positives through subsequent purification and re-screening (Figure 2 A & B). Several possible factors could have contributed to our inability to pick-up any positive clones, including: (i) low titer antibody, (ii) low level of expression of the EBS cDNA, and (iii) instability of the expressed protein in *E. coli*.

CONCLUSION

Taken together, the results of antibody characterization combined with those of cDNA library screen seem to suggest that the EBS antibody was of low titer. It may be EBS from rat, is not highly antigenic in mouse, a closely related species. Possibly, higher titer antibody could be generated using a different animal system, such as guinea pig or rabbit. This would of course require substantially more protein than was utilized in the production of antibody in mouse. A modified purification procedure, which would increase the yield of EBS polypeptide obtained, needs to be developed. Unfortunately, following this laboratory's move from UMAB to UMDNJ, a new source of pregnant rat uteri has not been identified. As type II EBS is only expressed at high levels under certain physiological conditions, such as estrogen stimulation or pregnancy, it is critical that the appropriate tissue be used. Future plans for this project are to attempt to induce expression of type II EBS in rat using estrogen implants. This would be followed by development of an improved purification procedure which may enable us to isolate the EBS gene. Once this is done it will be possible to address the goals described in the original proposal such as: expression of the EBS protein in *E. coli* and yeast, development of immunological and gene expression assays to be used to screen for the levels of EBS in normal and breast cancer tissues.

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APPENDICES

FIGURE LEGENDS

Figure 1: Western blot across size exclusion HPLC of type II EBS. Aliquots of the indicated fractions were resolved by 5---->15% SDS-PAGE, transferred to nitrocellulose and then probed with polyclonal anti-sera prepared against partially purified type II EBS. Detection was carried out using the ECL chemi-luminescent method (Amersham; Arlington Heights, IL).

Figure 2: Screening the rat uterine λgtII cDNA library. (A) Autoradiogram depicting the two positive clones obtained from initial screening of the cDNA library in λgtII. Arrows indicate clones which were isolated for purification and subsequent re-screening. (B) Autoradiogram depicting the absence of positive clones obtained by subsequent re-screen of the two positive clones obtained in (A).

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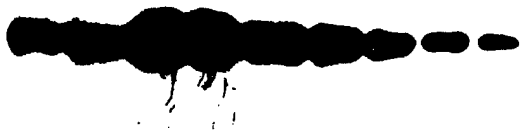

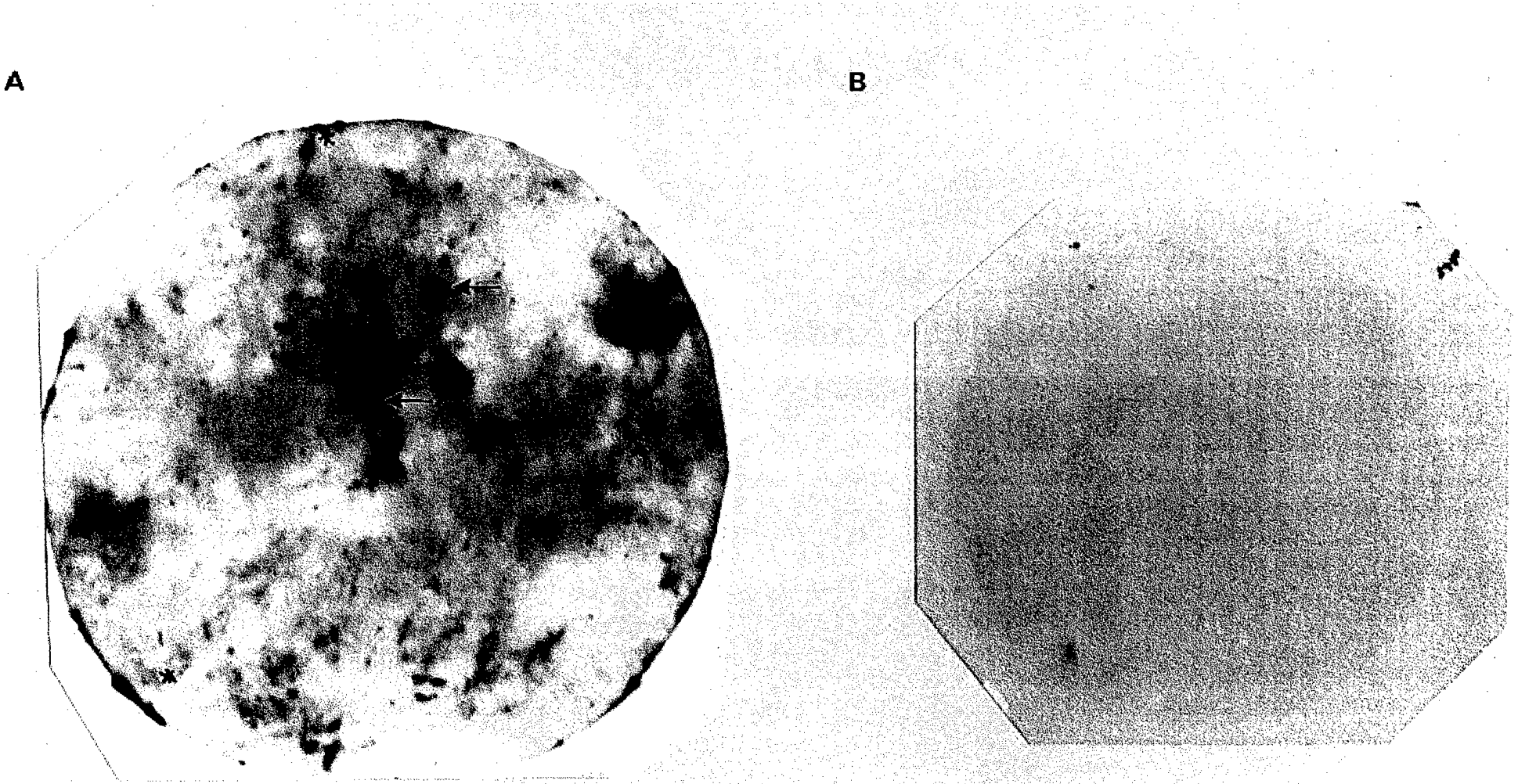
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